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Clinical and serological evaluation of a *Pasteurella haemolytica* A1 capsular polysaccharide vaccine

Jennifer A. Rice Conlon and Patricia E. Shewen*

The purified capsular polysaccharide (CPS) of Pasteurella haemolytica *A1 was examined for its ability to protect cattle from experimental challenge with logarithmic-phase* P. haemolytica. *Several preparations of* P. haemolytica *antigens were utilized in the experiment including CPS, log-phase* P. haemolytica *culture supernatant,* P. haemolytica *recombinant leucotoxin (rLKt) and various combinations of the above. CPS alone or in combination with culture supernatant or rLkt elicited no protection; rather, administration of CPS was associated with a high incidence of anaphylaxis (36% of calves). Although a classical biphasic humoral immune response to CPS could be detected in all calves that received this compound, this T-dependent response was not correlated with resistance to experimental challenge. The complexity of protective immunity in pneumonic pasteurellosis is emphasized by this study, and clinical anaphylaxis associated with response to CPS may be implicated in the pathogenesis of disease.*

Keywords: *Pasteurella haemolytica*; capsular polysaccharide; recombinant leucotoxin; T-dependent response

Bovine pneumonic pasteurellosis (shipping fever) is most often associated with isolation of *Pasteurella haemolytica* A1. This common disease of feedlot cattle is responsible for major economic losses to the cattle industry[1]. Various virulence factors have been associated with the organism and include capsular polysaccharide (CPS)[2–5], a ruminant-specific heat-labile leucotoxin (Lkt)[6,7], fimbria[1] and lipopolysaccharide[8–10]. Several of these antigens, including CPS, Lkt, sialoglycoprotease and neuraminidase, are present in bacterium-free culture supernatant harvested during the logarithmic-phase growth of the organisms. Vaccination of calves with this culture supernatant has been shown to induce resistance to experimental challenge[11–15]. As yet, the antigens necessary to stimulate a protective response against the disease are not fully understood.

Of the virulence factors described above, Lkt is the best characterized[6,16–24]. It is thought to play a role in pathogenesis by impairing primary lung defence and inducing inflammation[7,23,25]. While an anti-leucotoxin response is apparently crucial for disease resistance, this response alone does not confer immunity[12,15,21]. Vaccination with recombinant leucotoxin (rLkt) failed to protect cattle against experimental challenge with live organisms. However, enrichment of a commercial culture supernatant vaccine (Presponse, Langford Inc., Guelph, Ontario, Canada) with rLkt resulted in enhanced efficacy[11] indicating that other antigens in culture supernatant are also essential for disease resistance.

Several serotypes of *P. haemolytica* colonize the nasopharynx of healthy cattle[26]. However, serotype A1 predominates in recently shipped cattle and is the serotype most frequently isolated from pneumonic lungs. Since there appears to be a relationship between serotype-associated surface antigens and disease, we endeavoured to determine the protective efficiacy of one of the surface components unique to serotype 1, the CPS. We examined its efficacy alone, in combination with rLkt and as enrichment in a culture supernatant vaccine.

MATERIALS AND METHODS

Bacteria, plasmids and culture conditions

Escherichia coli HB101 containing the recombinant plasmids pLKT60 and pWAM716 have been described elsewhere[24]. Briefly, pLKT60 is a recombinant plasmid in which the Lkt structural genes, *lkt*C and *lkt*A, are placed behind the inducible *lac* promoter in the vector pTTQ18 (Ref. 27). pWAM716 was obtained from R.A. Welch (University of Wisconsin, Madison, WI, USA). It contains the *hly*B and *hly*D secretion genes of the related *E. coli* alpha-haemolysin determinant[28]. Preparation of recombinant rLkt has been described previously[11]. Logarithmic-phase cultures of *P. haemolytica* A1 (ATCC 43270) in brain–heart infusion broth were prepared for challenge as described by Shewen and Wilkie[15].

Capsular preparation

The purified capsular polysaccharide (CPS) of

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Table 1 Evaluation and scoring of clinical signs

Clinical sign	Score*
Nasal discharge	0.5
Cough	0.5
Dyspnoea	1.0
Off feed	
No hay	0.5
No hay or grain	1.0
Weak, lethargic	1.0
Down, unable to rise	1.0

*Maximum daily score = 5

P. haemolytica A1 was kindly provided by C. Adlam (Wellcome Biotechnology, UK)[2]. This purified product is protein-free by the Lowry assay standardized with bovine serum albumin, and was found to contain approximately 10 endotoxic units per mg by the *Limulus* amoebocyte lysate (LAL)) assay (Whittaker Bioproducts Inc., Walkersville, MD). This preparation runs in a polydispersed manner on SDS–polyacrylamide gel electrophoresis, and no staining is evident using Coomassie blue.

Vaccine preparations and trial design

Six groups of five and one group of four Holstein-Friesian calves ranging in age from 2 to 5 months were used in the trial. Each calf received one of seven vaccines intramuscularly twice with a 3-week interval. Three weeks after the last vaccination, all calves were challenged by the intrabronchial instillation of 25 ml of logarithmic-phase *P. haemolytica* A1 in phosphate-buffered saline (PBS) (absorbance at 525 nm (A_{525}) = 1; approximate concentration, 10^2 c.f.u. ml^{-1})[15]. Clinical signs were monitored and scored daily for 5 days prechallenge and 5 days postchallenge (*Table 1*)[15]. Six days after challenge, all calves were killed using intravenous barbiturate and the lungs were examined and scored for the percentage of pneumonic lung tissue[29].

The seven preparations used were PBS (group 1), the *P. haemolytica* culture supernatant vaccine Presponse (group 2), CPS (group 3), Presponse enriched with CPS (group 4), rLkt enriched with CPS (group 5), Presponse, rLkt and CPS together (group 6) and Presponse, mock rLkt and CPS (group 7). Mock rLkt[11] was used as a control for the effects, if any, of *E. coli* products such as endotoxin in the recombinant preparations, since such preparations contain $\approx 10^3$ endotoxin units per ml by the LAL assay. Presponse vaccine is endotoxin-free by the same assay. Vaccines for groups 2, 3 and 5 were given in 2 ml doses. Vaccines for groups 4, 6 and 7 contained 2 ml Presponse plus 2 ml CPS and rLkt (group 6) or mock Lkt (group 7). Group 1 calves received 4 ml PBS.

The rLkt and mock Lkt preparations were formulated as previously described[11]. Briefly, rLkt was used at a protein concentration of 3.2 mg ml^{-1}. Mock rLTK was used at a protein concentration estimated to represent the quantity of *E. coli* proteins present in the rLkt preparation. The capsular polysaccharide was used at 1 mg/dose reconstituted in PBS. Quil A (1 mg per calf; Cedarlane, Hornby, Ontario, Canada) in aluminium hydroxide (Cedarlane) was used as an adjuvant at an antigen-to-adjuvant ratio of 3:1 in all vaccine groups except group 1.

Serological evaluation

Blood samples were obtained from all calves in the vaccine trial on six separate occasions: day 1 (prevaccination), day 12, day 21 (at second vaccination), day 31, day 42 (prechallenge) and day 48 (at necropsy). Sera were stored at −20°C prior to analysis.

Enzyme-linked immunosorbent (EIA) assay

Isotype-specific (IgM, IgG_1, IgG_2, IgE) serum antibody responses to *P. haemolytica* A1 CPS were determined by EIA. All solutions were used at volumes of 100 μl unless otherwise stated. Optimal conditions consisted of the following: wells of 96-well microtitration plates (Nunc, Gibco, Missisauga, Canada) were coated with 10 μg of CPS in carbonate buffer (pH 9.6) overnight at 4°C. All plates were blocked for 30 min at 25°C with PBS containing 0.05% Tween 20 and 0.5% fish skin gelatin. This buffer was also used for all dilutions and washes unless stated otherwise. Four replicate wells of test sera at a dilution of 1:20 were incubated with antigen for 30 min at 36°C. After washing, biotinylated affinity-purified goat anti-bovine IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) at a 1:2000 dilution, monoclonal anti-bovine IgE (donated by L. Gershwin, Davis, CA, USA) at a 1:2000 dilution or IgG_1 or IgG_2 (Kirkegaard and Perry) at a 1:5000 dilution were incubated with the reaction mixture for 1 h at 37°C. Alkaline phosphatase-conjugated strepavidin (Kirkegaard and Perry) at a 1:2000 dilution in PBS was added and incubated for 40 min at 37°C. Colour was developed with *p*-nitrophenylphosphate. A known positive serum and fetal bovine serum at a dilution of 1:20 were used as positive and negative controls, respectively. Absorbance (A_{405}) of the samples was read and readings were considered valid if the coefficient of variation (CV) of four replicate wells of positive control serum was $\leqslant 15\%$. The plates were standardized from day to day by reading the plates when the absorbance of the positive control sample gave an approximate reading of 1.0. The results were then expressed as ratio of the absorbance of the sample to the absorbance of the control.

SDS–polyacrylamide gel electrophoresis and Western immunoblot analysis

The capsular polysaccharide of *P. haemolytica* was subjected to SDS–polyacrylamide (12%) gel electrophoresis[30] and transferred to nitrocellulose paper. Antisera from calves exhibiting signs of anaphylaxis (reactor calves) (day 0 and day 21) and antisera from calves not exhibiting anaphylaxis (non-reactor calves) were used at a dilution of 1:100 to probe the nitrocellulose. Biotinylated monoclonal anti-bovine IgE was used as the second antibody followed by incubation with alkaline phosphatase-conjugated strepavidin. Colour was developed using nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indoly phosphate (BCIP) and *N,N*-dimethylformamide in carbonate buffer (pH 9.8).

Statistical analysis

Clinical score and post mortem data were analysed by the Kruskal–Wallis technique using a non-parametric paired comparison[31], while serological data were

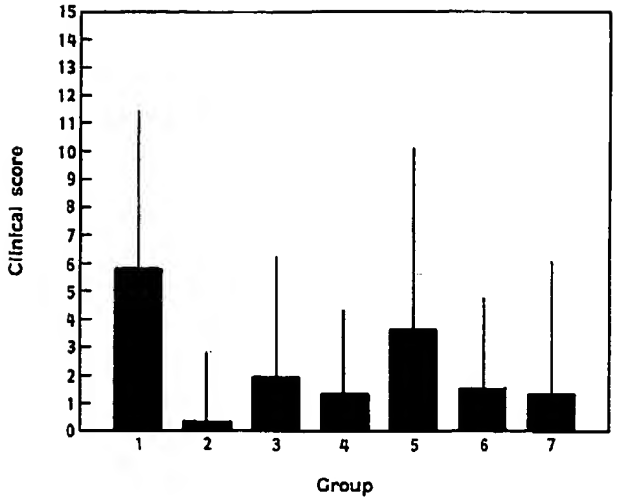

Figure 1 Group mean clinical score during 5 days postchallenge. Maximum score = 25 (maximum daily score = 5; $n = 5$, groups 1, 2, 3, 4 and 5; $n = 4$, groups 6 and 7). Clinical scores were zero for all calves prechallenge. Lines represent standard deviation within each group. No significant differences were found between vaccine groups

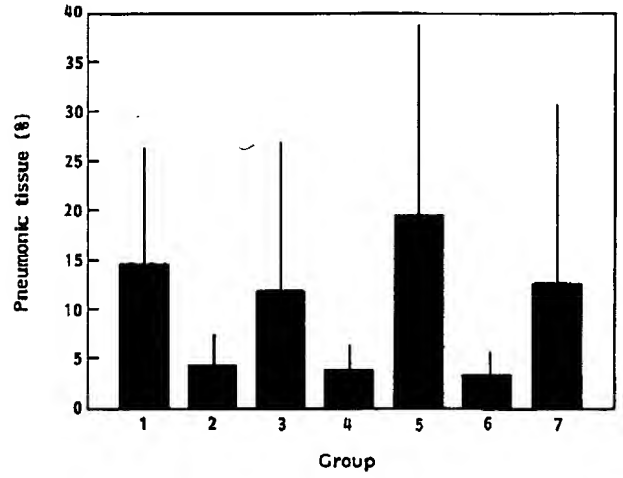

Figure 2 Group mean percentage pneumonic tissue. Lines represent standard deviation within each group. No significant differences were found between vaccine groups ($n = 5$, groups 1, 2, 3, 4 and 5; $n = 4$, groups 6 and 7)

analysed by the GLM procedure (SAS, Cary, NC, USA). The probability level for significance was at least 95%.

RESULTS

Evaluation of clinical scores

The mean 5-day clinical score postchallenge for each of the groups is shown in *Figure 1*. No significant difference among groups was evident. Nine of the 25 calves that received a vaccine containing purified *P. haemolytica* CPS alone or with other antigens developed profuse sweating, panting, dilation of pupils, diarrhoea, collapse, and in one case death. These signs, which were typical of a classical anaphylactic reaction, occurred at the time of initial vaccination in one calf and at second vaccination in eight calves. This represents a large proportion of the calves (36%) that received CPS. Except in the first case, administration of epinephrine ameliorated the condition.

Evaluation of post mortem findings

The mean percentage pneumonic tissue for each group is shown in *Figure 2*[29]. Differences among groups were not significant.

Capsule-specific EIA results. Isotype-specific (IgM, IgE, IgG_1 and IgG_2) analyses for anti-capsular antibodies were performed on all sera collected during the trial. No reactivity of the IgE isotype was found. For IgM, IgG_1 and IgG_2, prevaccination anti-capsule titres were not significantly different between groups (*Figures 3, 4* and *5*). By 10 days after the first vaccination, a significant ($p < 0.0015$) increase in capsule-specific IgM titres was observed in vaccinated calves. This increase

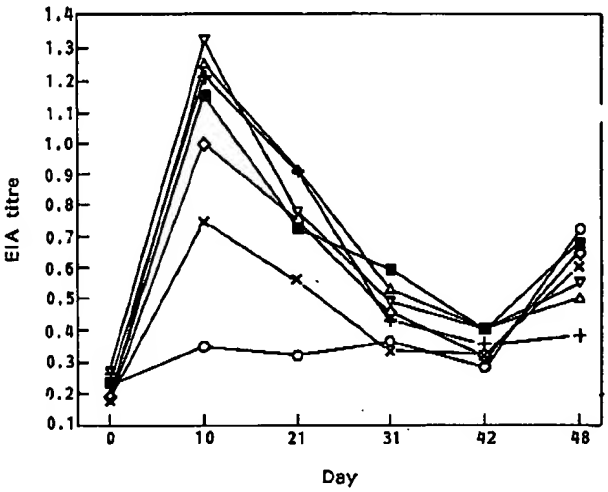

Figure 3 Group mean IgM capsule-specific EIA titre, defined as a ratio to positive control serum. Serum samples were collected on day 0 (prevaccination), day 12, day 21 (second vaccination), day 31, day 42 (challenge) and day 48 (post mortem). ○, Group 1; ■, group 2; +, group 3; ◇, group 4; △, group 5; ×, group 6; ▽, group 7

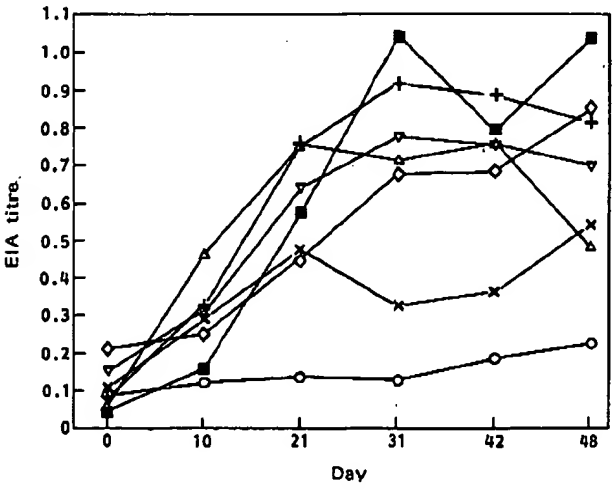

Figure 4 Group mean IgG_1 capsule-specific EIA titre, defined as a ratio to positive control serum. Serum samples were collected on day 0 (prevaccination), day 12, day 21 (second vaccination), day 31, day 42 (challenge) and day 48 (post mortem). ○, Group 1; ■, group 2; +, group 3; ◇, group 4; △, group 5; ×, group 6; ▽, group 7

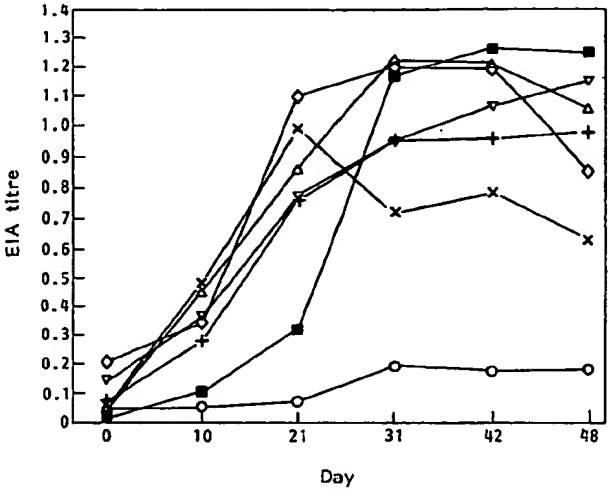

Figure 5 Group mean IgG_1 capsule specific EIA titre, defined as a ratio to positive control serum. Serum samples were collected on day 0 (prevaccination), day 12, day 21 (second vaccination), day 31, day 42 (challenge) and day 48 (post mortem). ○, Group 1; ■, group 2; +, group 3; ◇, group 4; △, group 5; ×, group 6; ▽, group 7

was not evident in either the IgG_1 or the IgG_2 isotypes. By day 21 (at second vaccination), both the IgM and IgG_2 titres were not significantly different among the groups; however, a significant increase in IgG_1 ($p > 0.0004$) titre was seen in groups 2, 3, 4, 5 and 7. Calves that received Presponse enriched with rLkt and CPS (group 6) showed no significant rise in IgG_1 titres compared with the PBS controls. On the day of challenge (42 days after first vaccination and 21 days after second vaccination), capsule-specific IgG_1 tritres in groups 2, 3, 4, 5 and 7 were significantly higher than those in the control group. However, no significant differences were found in the capsule-specific IgM or IgG_2 titres among groups. There was no correlation between lung lesion scores and EIA titre to capsular polysaccharide in any of the isotypes.

Western immunoblot analysis

The IgE-specific Western immunoblot analysis of the capsular polysaccharide is presented in *Figure 6*. Lane 1 (day 0) and lane 2 (day 21) are immunoblots using sera obtained from a calf receiving Presponse whereas lane 3 (day 0) and lane 4 (day 21) are immunoblots using sera obtained from a calf receiving CPS alone. Neither of these calves demonstrated signs of anaphlyaxis (non-reactor calves). These Western immunoblots represent the strongest response seen of the various non-reactor calves tested. Lanes 5 to 12 are immunoblots using sera collected from calves demonstrating clinical anaphylaxis (lanes 5, 7, 9 and 11 are blots using sera collected prevaccination and lanes 6, 8, 10 and 12 are blots using sera collected on day 21 when clinical anaphylaxis occurred). Increased reactivity to a band of approximately 96 kDa was evident in the samples taken from the reactor calves on day 21. This was only weakly recognized by serum from a non-reactor calf (lanes 1 and 2).

DISCUSSION

For many years, the CPS of *P. haemolytica* A1 has been considered to be an important virulence factor[4,5], perhaps explaining the predominance of serotype A1 in disease. Nevertheless, previous studies in rabbits and young lambs have shown that it is poorly immunogenic. Although CPS did elicit an antibody response when administered in oil adjuvant to mature cattle and sheep[2], the response was relatively weak, suggesting that CPS in its purified form is a poor immunogen. However, our study shows that CPS in a Quil A/aluminium hydroxide adjuvant is a good immunogen in cattle. Contrary to the notion that purified CPS acts in a T-independent manner[32], we observed a classical biphasic humoral response with isotype switching from IgM to IgG_1 and IgG_2. Such diversity of response has been reported when purified CPS of other organisms has been conjugated to a protein carrier[33–36]; yet, in our study, no such antigen manipulation was employed. This rather intriguing observation may reflect the influence of *P. haemolytica* as a commensal in the nasopharynx of healthy cattle. Although not actively responding to CPS prior to vaccination, these cattle, like others in the field, are not naive and have been primed to CPS by previous natural exposure to the intact live organism. This observation has important implications for the development of component vaccines against enzootic organisms, and underscores the relevance of using conventional (as opposed to artifically reared) subjects of the target species in developmental trials. Interestingly, the poor immunogenicity of many of the above referenced CPS vaccines is related to their use in human infants who are likely to be naive to colonization by the organism. Thus, age-matched trials may also be necessary for proper vaccine development.

The presence of an IgE response on Western immunoblot to a component of the purified CPS may indicate a *P. haemolytica* antigen associated with anaphylaxis. It is interesting that one calf vaccinated with the commercial culture supernatant vaccine (Presponse) also had weak reactivity to this 96 kDa antigen. This would suggest that a weak IgE response may occur in response to this pathogen, but when present in high concentration, this response is associated with clinical and systemic anaphylaxis. The inability to detect the presence of IgE by EIA may be associated with hidden

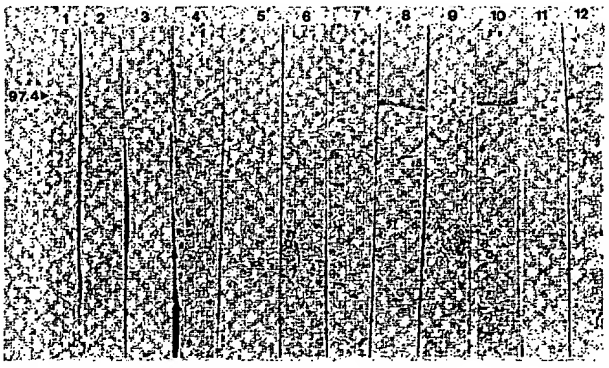

Figure 6 IgE-specific Western blot analysis of *Pasteurella haemolytica* A1 capsular polysaccharide. Lane 1 (day 0) and lane 2 (day 21) are blots from a non-reactor calf (no clinical signs of anaphylaxis) receiving Presponse as a vaccine; lane 3 (day 0) and lane 4 (day 21) are blots from a non-reactor calf receiving purified CPS as a vaccine; lanes 5 to 12 represent blots from reactor calves (clinical signs of anaphylaxis). Odd-numbered lanes are samples taken prevaccination (day 0) and even-numbered lanes are samples taken at second vaccination (day 21)

antigenic determinants which are exposed for reactivity only after boiling and electrophoresis of the compound as for Western immunoblots. Alternatively, the concentration of IgE reacting in the EIA may be insufficient for detection by the methodology utilized in this study.

The lack of correlation between CPS titre and protection is most probably associated with the high variability in lung lesion scores within the vaccine groups. This differs from the findings of Confer and co-workers who were able to demonstrate a correlation between antibody response to purified CPS and resistance to *P. haemolytica* challenge[37]. Unpublished work from this laboratory suggests a strong association between naturally occurring titres and the season of the year. Previously published vaccine trials which demonstrated protection using some of the vaccine groups also included in this study were performed at a different time of year[11]. Although not statistically different, trends in the protection seen with the various vaccines used in this study are similar to those presented in previous work[11]. Variability may also be attributed to the clinical or subclinical effects of pulmonary anaphylaxis in the vaccine recipients which would confound the lesion scoring at the time of necropsy. The high percentage (36%) of calves that developed anaphylaxis upon receiving the CPS preparation would negate the usefulness of this antigen as a vaccine candidate. Interestingly, Pasteurella bacterins used extensively in the past induced anaphylaxis in some vaccinated animals. This was assumed to be mediated by the endotoxin component of the *P. haemolytica* cell wall. In this study, all calves that showed an anaphylactic response had received CPS alone or with other *P. haemolytica* antigens, but with relatively low amounts of *P. haemolytica* endotoxin. These observations suggest that the CPS may be the component responsible for the anaphylactic response associated with killed bacterin vaccines.

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